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Transcriptional repression of the tumor suppressor DRO1 by AIB1

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ABSTRACT

Using transcriptomic gene expression profiling we found tumor suppressor DRO1 being repressed in AIB1 transgenic mice. In agreement, AIB1 represses DRO1 promoter and its expression levels inversely correlate with DRO1 in several cancer cell lines and in ectopic and silencing assays. Estrogen modulators treatment showed a regulation in an estrogen receptor-dependent fashion. Importantly, DRO1 overexpression resulted in BCLAF1 upregulation, a compelling concept given that BCLAF1 is a death-promoting transcriptional repressor. Additionally, DRO1 shuttles from Golgi to the endoplasmic reticulum upon apoptotic stimuli, where it is predicted to facilitate the apoptosis cascade. Finally, DRO1 repression is an important factor for AIB1-mediated inhibition of apoptosis. Collectively, our results reveal DRO1 as an AIB1-targeted tumor suppressor, providing a novel mechanism for AIB1-dependent inhibition of apoptosis.
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1. Introduction

Improper balance between cell proliferation and apoptosis is a hallmark of cancer. During tumor progression, oncogene activation combines with loss of suppressor function to escape from senescence and apoptosis, providing advantages to affected cells. This combination can be acquired in sequential steps or directly by a single modification that inhibits compensatory tumour-suppressive responses. Elucidating the pathways underlying an oncogene or tumor suppressor is a key to find new strategies for improving cancer therapy.

The gene coiled-coil domain containing 80 (i.e. CCDC80, also called DRO1, URB, SSG1 and equarin) was identified as upregulated in brown adipose tissue of mice deficient in bombesin receptor subtype-3 [1]. Further studies revealed to be abundantly expressed in fat and deregulated in obesity [2], regulating adipogenesis through the down-regulation of Wnt/β-catenin signalling and induction of C/EBPα and PPARγ [3]. In addition, DRO1 is also expressed in dermal papilla cells [4], in bone marrow stromal cells and during development suggesting a role in skeletogenesis [5] and in eye formation [6]. The expression of DRO1 mRNA was also identified as down-regulated by 17β-estradiol in the rat uterus [7], although protein was increased in the mammary gland of treated rats. However, the molecular weight of the protein analyzed in these experiments was rather too low, about 40 kDa, indicating the crossreactivity of antibodies with other molecular species or the detection of a subproduct. Interestingly, DRO1 was found to sensitize cells to induced apoptosis and its expression was highly reduced in colon and pancreatic cancers [8], revealing its role as a tumor suppressor.

AIB1 is an oncogene that functions as a transcriptional coactivator in the nucleus [9,10]. Nuclear shuttling of AIB1 correlates with cell cycle progression in non-cancer cells [11], but this distribution is altered in cancer cell lines [12]. AIB1 overexpression occurs in many types of tumors (reviewed in [13]) and this is also the cause of cancer in animal models [14]. Noteworthy, an oncogenic isoform of AIB1 lacking the nuclear localization signal (AIB1-Delta3) localizes in the cytosol and controls cytoskeletal organization and cell migration [15]. Most of the work done to characterize AIB1 oncogenic-dependent pathways has lead to the identification of other oncogenes that help to explain AIB1 pathology. High levels of AIB1 stimulate invasiveness and correlates with high levels of Twist [16]. In addition, AIB1 promotes G1 progression by coactivating the transcription of E2F1 [17]. AIB1 also functions as an AP-1 coactivator to upregulate the expression of MMP7 and MMP10 in breast cancer cell lines [18]. Similarly, in prostate cancer cell lines AIB1 overexpression enhances coactivation of the transcription factors PEA3 (an Ets family member) and AP-1, thus increasing the levels of MMP2 and MMP13 and resulting in greater ability to migrate [18]. These different expression patterns suggest that AIB1-induced MMP expression depends on the cellular context. Moreover, AIB1 regulates the expression of MMP2 and MMP9 through PEA3 transactivation activity [19]. AIB1 has also been associated with ER81, another member of the Ets family, to stimulate the expression of

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MMP1 [20]. These results strongly support the idea that AIB1 overexpression can promote cancer initiation and progression through the synergistic action of additional alterations. However, little is known about the link between AIB1 and tumor suppressors.

2. Materials and methods

2.1. Cell cultures and procedure

Human mammary epithelial cells (HMEC, Cambrex) were grown in MEBM media supplemented with MEGM growth requirements (Clonetics). All other cell lines were grown in DMEM supplemented with 10% FBS. HMECs were infected at 40 MOI with Ad5-adenovirus to express human AIB1 or green fluorescent protein (GFP) as control. Fourty nanometers Stealth siRNA (Invitrogen) targeting AIB1 or DRO1 mRNA were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. DNA transfections were performed with Lipofectamine LTX reagent (Invitrogen) according to company specifications. Cells were harvested 48 h post-transfection or infection. For the stimulation with estrogen modulators, MCF-7 cells were grown for a week in DMEM phenol-red-free medium plus 10% charcoal and dextran-treated FBS (HyClone). At time 0 cells were stimulated either with 10 nM 17β-estradiol, 10 μM 4-hydroxytamoxifen (Sigma) or 1 μM ICI 182780 (AstraZeneca Pharmaceuticals). DRO1 harboring the Flag epitope at the C-terminus was cloned under the doxycycline-inducible promoter pTRE2hyg and transfected in Tet-On inducible MCF-7 cells (Clontech). Cells were grown in the presence of 100 μg/mL G418 (Sigma) and 100 μg/mL hygromycin B (Invitrogen). DRO1 expression was induced with 10 μg/mL doxycycline (Sigma) at least 48 h before each experiment.

2.2. Reverse transcription and quantitative real-time PCR

Total RNA was isolated using TRIzol (Invitrogen) reagent. For BCLAF1 cDNA synthesis followed by quantitative real-time PCR (qRT-PCR) was performed following manufacturer's protocol (SABioscience RT2 qPCR) using HPRT1 as housekeeping gene. For the rest of genes, cDNA was synthesized with SuperScript First-Strand (Invitrogen) and quantified with a standard SYBR green PCR kit (Eurogen). Primers used were: DRO1 5′ TATGTGCAACAACGTGATGA 3′ and 5′ AAGTGGTCGATTTTCATGGT 3′; AIB1 5′ GGCCAGTGATTCACGAAAACG 3′ and 5′ ACTTTCCTGCTCCCGTCTCC 3′; GAPDH as housekeeping gene 5′ ACCACAGTCCATGCCATCAC 3′ and 5′ TCCACCACCCTGTTGCTGTA 3′.

2.3. Cell apoptosis analysis by flow cytometry

MCF-7-TetOn cells were treated for 6 h with 1 μM staurosporine (Sigma) to induce apoptosis. Cells were trypsinized, washed with PBS and 250×10^3 cells were centrifuged at 200*g* for 5 min and further fixed in 1 ml 70% ethanol at −20 °C for 16 h. Cells were stained overnight in 250 μL PBS containing 50 μg/mL propidium iodine and 1 ng/mL RNase (Roche) and analyzed in a Cytomics FC500 (Beckman Coulter). TUNEL assay was performed with cell death detection kit (Roche). Staurosporine was maintained for 19 h in cells analyzed by flow cytometry.

2.4. Vector construction and luciferase assays

DRO1 cDNA was amplified from HeLa cells by RT-PCR with oligos 5′ ACACAATGACATGGAGAATGG 3′ y 5′ GCAGCTGCAGAGGAAACTG 3′ and Flag sequence was further added at the C-terminus

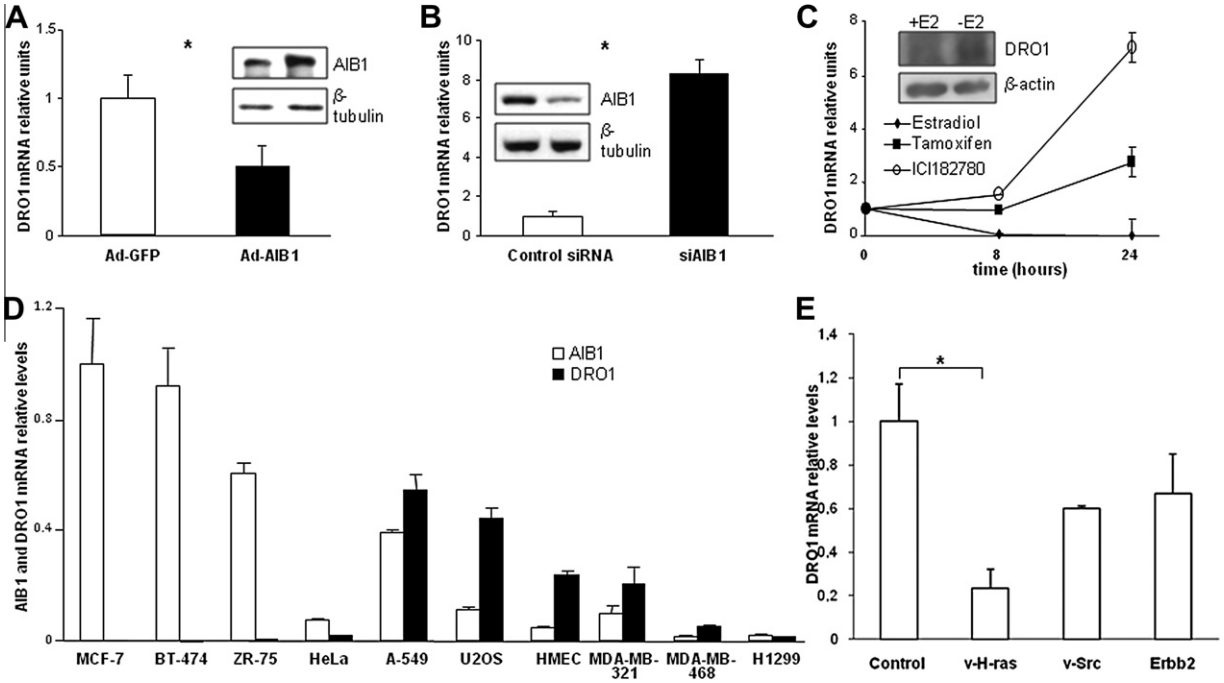

Fig. 1. AIB1 negatively regulates DRO1 mRNA levels. (A) HMEC were infected with adenovirus expressing vectors (Ad-GFP, □; Ad-AIB1, ■) and two days later relative DRO1 mRNA levels were quantified by qRT-PCR. Values were normalized against GAPDH levels and represent the mean value of three experiments ± standard error (SE). $^*P < 0.01$. (B) MCF-7 cells were transfected with control siRNA (□) or with specific AIB1 siRNA (■). Relative DRO1 mRNA levels were quantifyed 2 days later. Values represent the mean of three replicates from three independent experiments ± SE. Western blotting in the insets show AIB1 levels for each of the conditions tested. (C) MCF-7 cells were estrogen deprived for a week and further stimulated with 10 nM 17-β-estradiol. Total RNA was isolated at the indicated times and relative DRO1 mRNA levels were determined. Inserts are western blots against DRO1 and β-actin as loading control of MCF-7 cells grown in the presence (+E2) or absence (−E2) of estradiol. Mean values of three experiments ± SE. (D) Different human cancer cell lines and primary non-cancerous HMEC were grown under standard conditions. DRO1 mRNA levels (■) and AIB1 levels (□) were quantified by qRT-PCR and normalized against GAPDH according to Pfaffl's equation. Bars represent mean values of four replicates ± SE. (E) DRO1 mRNA levels were determined in U2OS cells transfected with 500 ng vector alone (Control) or containing either of the oncogenes v-H-ras, v-Src or Erbb2. Bars are mean values of four replicates ± SE. Significative values are indicated $^*P = 0.016$.

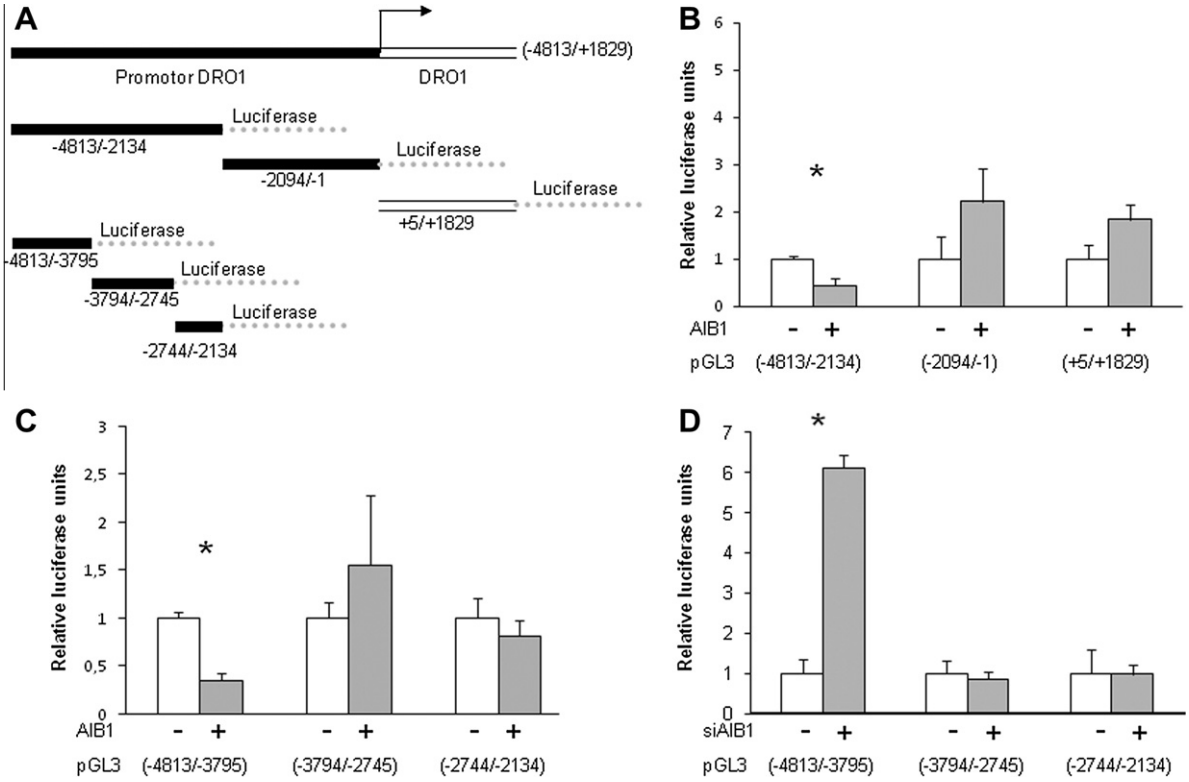

Fig. 2. Transcriptional regulation of DRO1 promoter by AIB1. (A) Schematic representation of DRO1 promoter and reporter constructs. Arrow indicates the transcriptional start site; black box corresponds to the promoter region and white box corresponds to transcribed DRO1 that includes the first intron and part of the second exon. (B) COS1 cells were transfected with the indicated genomic regions of DRO1 gene and cotransfected with AIB1 or pCDNA3.1 empty vector. Luciferase activities were normalized for transfection efficiency by renilla activities. Values represent fold activation over vector without AIB1. $^{*}P = 0.02$. (C) Fragments of the distal promoter region were obtained by PCR and analyzed as in (B). Values represent fold activation over vector without AIB1.$^{*}P = 0.01$. (D) MCF-7 cells were silenced with interference RNA for AIB1 (+) or RNAi control (−) and one day later were transfected with same reporter constructs as in (C). Two days later luciferase assay was determined as in (B). Values represent fold activation over control RNAi. $^{*}P = 0.0001$.

by PCR with oligos 5′ GCGCCTCGAGATGACATGGAGAATGGGACCC 3′ and 5′ GCGCGAATTCTCACTTGTCGTCATCGTCCTTGTAGTCGTAAGGGTATCCATG 3′. The resulting amplicon was cloned into XhoI/EcoRI sites of pcDNA3.1- and sequenced for validation. DRO1 promoter regions were amplified from U2-OS genomic DNA with the following oligos: (−4813, −2134) 5′ CTACAAGGTTTCCCCAGCAA 3′ and 5′ GGGCTTCAGAAAATCCTCCT 3′; (−2094, −1) 5′ AAGCCCCACTTTGCCAGTGT 3′ and 5′ GAGTGAGAAGGCTGTTCTTTG 3′; (+5, +1829) 5′ CTGAGTCCACTCTGAACGTGCT 3′ and 5′ TTCTCACCTTGCCTCCTTCCT 3′ and subcloned as KpnI/NheI; NheI/BglII and BglII/HindIII fragments, respectively in the pGL3 basic Luciferase vector (Promega). One day before transfection 50×10^3 COS1 cells were seeded in sextuplicates in six well plates. Each transfection contained 500 ng pGL3 basic vector harbouring each of the promoter inserts or empty vector, 100 ng pcDNA 3.1-AIB1 or empty vector and 30 ng pRL-TK as internal control. Luciferase and renilla assays (Promega) were done 48 h after transfection.

2.5. Western blot and immunofluorescence

Cell lysates were done with commercial lysis buffer (Cell Signaling) supplemented with protease cocktail inhibitors (Roche), 150 mM NaCl, 1 mM AEBSF (Roche), and 1 mM NaF. Lysates were centrifuged at 14,000 rpm for 15 min. Thirty micrograms of protein was resolved on SDS–PAGE gel and transferred to PVDF membranes. For immunofluorescence, cells were grown on cover slips. Colocalization with subcellular compartments was performed with Organelle lights (Invitrogen) 24 h after baculovirus infection. Cells were fixed, and stained with anti-Flag antibodies (Sigma) followed by anti-mouse antibodies coupled to Cy3 (Jackson ImmunoResearch), mounted with fluorescent mounting media (Dako) and visualized with a fluorescent microscope. DRO1 antibodies were generated by immunizing rabbits with peptides corresponding to amino acids 313–328 and 935–950 of human DRO1 sequence.

3. Results and discussion

3.1. Down-regulation of DRO1 by AIB1

Most of the work done to explain AIB1 oncogenic potential has lead to the intervention of other oncogenes and oncogenic pathways. The mechanisms of AIB1 to promote cancer initiation, progression, and endocrine resistance involve a variety of signaling pathways, including ER, IGF/PI3K/AKT, HER2, NF-κB and Ets as well as cell cycle regulation (reviewed in [21]). AIB1 is also an in vivo transducer of H-*Ras*-induced tumors [22]. Using high-density genome-wide expression microarrays to analyze the differential expression of genes we detected a significant down-regulation of DRO1 in transgenic mouse mammary epithelial cells overexpressing AIB1 (Fig. S1 and Tables S1 and S2). Since DRO1 has been reported to be a tumor suppressor with pro-apoptotic characteristics, we thought this finding of interest and first focused to demonstrate the array results. Overexpression of AIB1 in human primary cultures (HMECs) potently inhibited DRO1 mRNA levels (Fig. 1A), supporting the role of AIB1 as a negative regulator for DRO1. In addition, silencing the expression of AIB1 in MCF-7 cells, a human mammary carcinoma that overexpresses AIB1, resulted in a significant increase in DRO1 levels (Fig. 1B), suggesting that DRO1 is repressed in MCF-7 by AIB1. Moreover, stimulation of

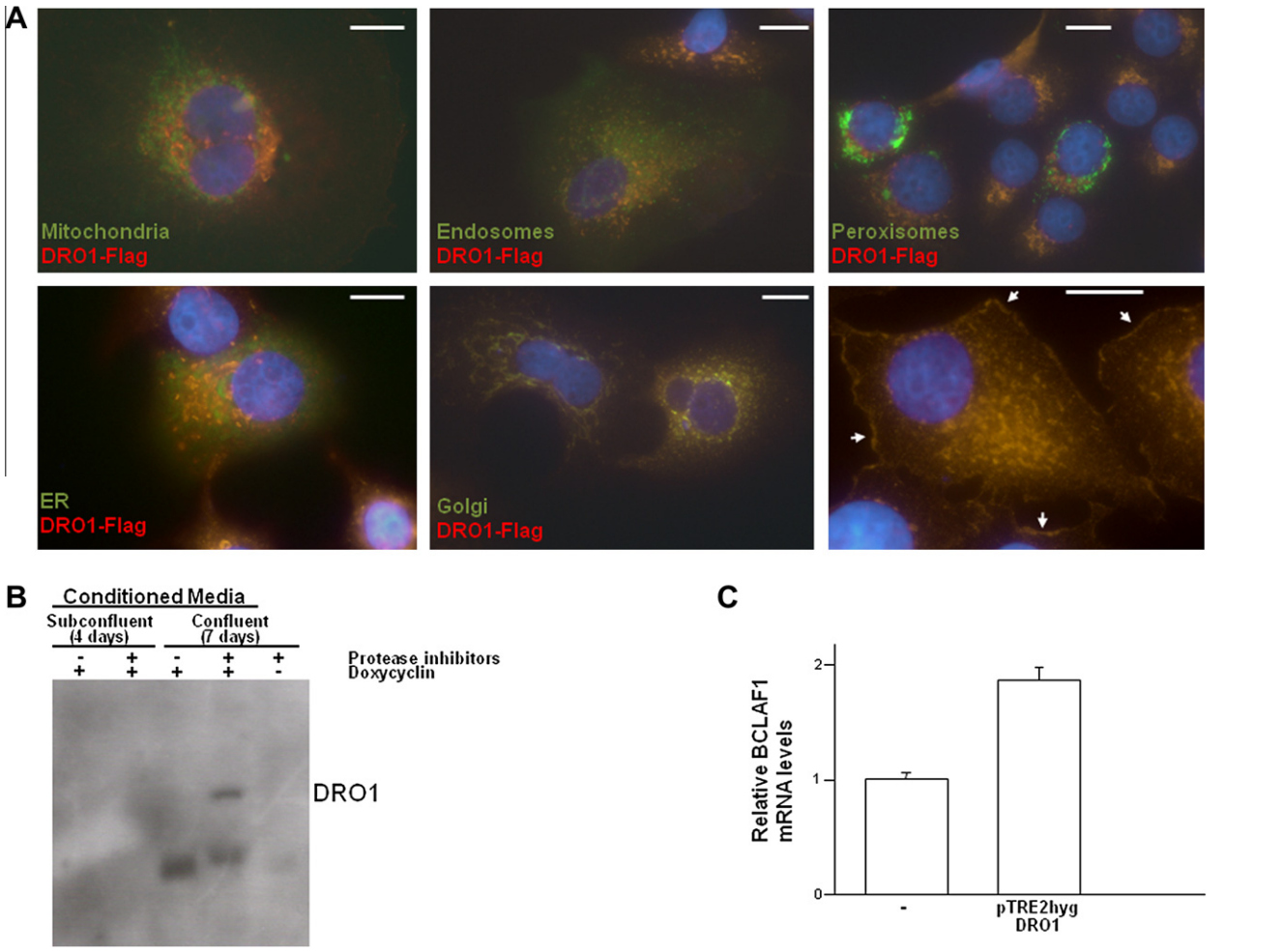

Fig. 3. Ectopic expression of DRO1 co-localizes with Golgi compartment and cellular perimeter. (A) Expression of Flag-DRO1 was induced by doxycycline treatment in Tet-On inducible MCF-7 cells. Cultures were further infected with GFP constructs targeting specific subcellular structures as indicated in each panel. DRO1 was visualized with anti-Flag antibodies, followed by incubation with Cy3-conjugated donkey anti-mouse antibodies. Scale bar: 10 μm. Flag-DRO1 staining around the perimeter of the cell is indicated with arrows. (B) Conditioned media from doxycycline-stimulated cells for the indicated times were analyzed by western blot with DRO1 antibodies. Protease inhibitors (Roche) were added (+) or not (−) for the last 24 h to prevent DRO1 proteolysis and potentiate DRO1 signal [3]. (C) Fold increase in BCLAF1 expression was determined after doxycycline treatment for 3 days in MCF-7 cells harboring the DRO1 inducible vector (pTRE2hygDRO1) or in the parental cell line lacking it (−). Bars represent mean values of three independent experiments ± SE, $P < 0.05$.

MCF-7 cells with 17-β-estradiol reduced DRO1 expression levels through time, whereas treatment with anti-estrogens (tamoxifen or ICI182780) increased DRO1 levels (Fig. 1C). These results suggest that AIB1 as well as the estrogen receptor repress DRO1 and confirm our initial findings in AIB1 transgenic mice.

We further quantified DRO1 expression in different cancer cell lines and compared them to AIB1. Although there are big variations in DRO1-expression levels among the different cancer cell lines tested, we noticed that many of them show an inverse correlation between AIB1 and DRO1 expression levels (Fig. 1D). In general, these data further support a new role for AIB1 as a negative regulator of DRO1 expression. DRO1 is repressed in colorectal carcinomas [8] and AIB1 overexpression correlates with clinical stage of human colorectal carcinoma [23]. However, the lung cancer cell line H1299 shows very low levels of either of them and the ratio in A-549 is also lower than in other cell lines, suggesting that DRO1 expression is also affected by genes other than AIB1. In fact, DRO1 expression was markedly decreased after neoplastic transformation by β-catenin or K-Ras [8]. In agreement with the down-regulation of DRO1 by oncogenes, transfection of v-H-Ras, v-Src and Erbb2 resulted in decreased DRO1 levels (Fig. 1E), confirming that multiple oncogenes modulate DRO1 expression. Based in DRO1 pro-apoptotic function, our results support that DRO1 may constitute a novel mechanism to explain AIB1 inhibition of apoptosis [14].

3.2. Promoter analysis of DRO1

To further study and map DRO1 regulatory elements affected by AIB1, we cloned sequential regions of its promoter and analyzed them in a luciferase reporter assay. As shown in the schematic representation in Fig. 2A, we evaluated a distal region to the start of transcription (−4813, −2134); a proximal region (−2094, −1) and a transcribed region containing the first intron (+5, +1829). The last two regions showed a relatively higher activity when cotransfected with AIB1 (Fig. 2B), which may be due to the presence of several responsive elements for transcription factors for which AIB1 is a coactivator. For instance, there are several potential binding sites for NF-κB and Pax-2. Noteworthy, the distal promoter region displayed a significant decrease in the transcriptional activity when cotransfected with AIB1, supporting to contain the regulatory elements that down-regulate DRO1. Further promoter analysis with more detailed constructs to nail down the region of interest delimited AIB1 regulatory domain to (−4813/−3795) (Fig. 2C). Conversingly, silencing AIB1 in MCF-7 cells resulted in increased reporter activity when transfected specifically with

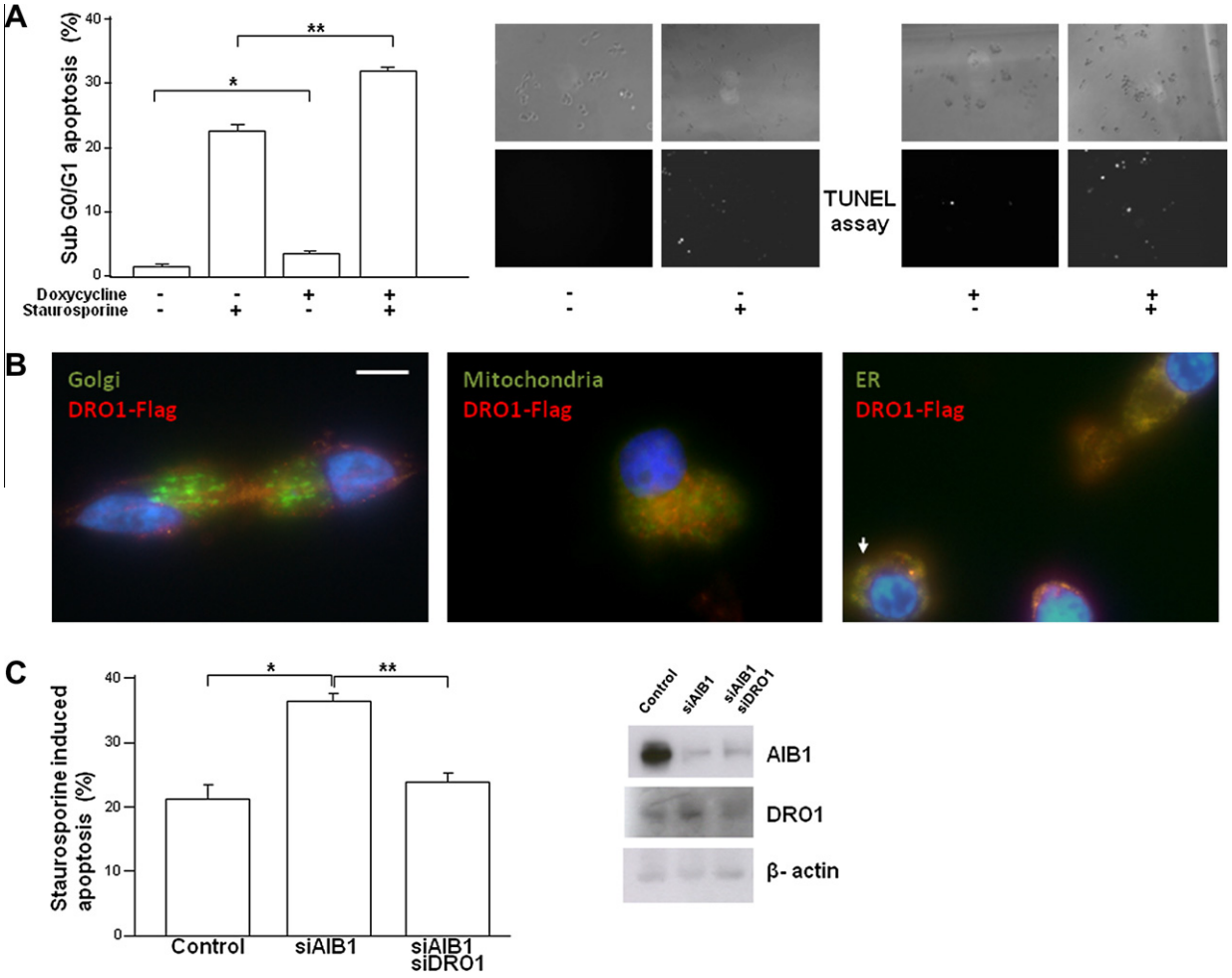

Fig. 4. Induced apoptosis promotes DRO1 translocation to the endoplasmic reticulum. (A) Subconfluent Tet-On inducible MCF-7 cells were grown in the presence (+) or absence (−) of 10 μM doxycyclin for 4 days to induce DRO1 expression. Cells were further treated with 1 μM staurosporine (+) for 6 h to induce apoptosis. Cultures were analyzed by flow cytometry and the percentage of the sub-G0/G1 fraction was quantified to estimate the ratio of apoptosis (graphic on the left). Bars represent mean values of three replicates ± SE, $^{*}P = 0.002$; $^{**}P = 0.001$. Increase in apoptosis after staurosporine treatment and/or induced DRO1 expression was also confirmed by TUNEL (bottom panels right). (B) GFP-expressing cells in different subcellular compartments reveal translocation of DRO1 from Golgi to the endoplasmic reticulum after staurosporine treatment. (C) AIB1 expression alone or in combination with DRO1 was silenced as indicated in MCF-7 cells. Cells were further treated with staurosporine and sub-G0/G1 population was calculated by flow cytometry and referred to levels without staurosporine. Bars represent mean values of three independent experiments ± SE, $^{*}P = 0.004$; $^{**}P = 0.002$. Western blot analysis was done in parallel to demonstrate AIB1 and DRO1 expression levels under the same conditions.

domain (−4813/−3795) (Fig. 2D), thus supporting to be the regulatory domain for AIB1.

3.3. DRO1 colocalizes in Golgi and at the plasmatic membrane

Previous studies indicated that DRO1 is a secreted protein [3,5,6] that is also found in the extracellular matrix [24]. However, these results are partially controversial with other authors that localized DRO1 in the cytosol [7] or that could not detect DRO1 at the membrane or weakly in the secretory pathway [8]. To resolve this question we generated a doxycycline inducible cell line for the expression of DRO1. MCF-7-TetOn cells expressing DRO1-Flag were further infected with viruses to express GFP specifically at selected subcellular compartments. Our results in Fig. 3 clearly demonstrate that DRO1 is localized at the Golgi apparatus. When induction of DRO1-Flag expression was prolonged for 10 days, DRO1 could also be detected at the cytoplasmic membrane (Fig. 3A, right bottom panel). DRO1 was also detected in the conditioned media 7 days after doxycycline treatment (Fig. 3B), thus confirming to be a secretory protein that can also be retained at the cell surface when overexpressed. In addition, based in previous report supporting a role for DRO1 in apoptosis [8] we performed a PCR array analysis (RT2 Profiler Human Apoptosis, PAHS-012F-2; SABiosciences). Importantly, overexpression of DRO1 resulted in significant increase of BCLAF1 expression (Fig. 3C), which may explain in part the increased sensibility to apoptotic stimuli when DRO1 is overexpressed [8].

3.4. AIB1 inhibition of apoptosis is partly dependent in DRO1 repression

In order to study the contribution of DRO1 in apoptosis we analyzed DRO1-Flag inducible cells after staurosporine treatment. Staurosporine induced highest apoptosis in DRO1-Flag expressing cells, as determined by TUNEL assay and in sub-G0/G1 population by flow cytometry analysis (Fig. 4A). Interestingly, ectopic expression of DRO1 colocalizes abundantly in Golgi but is potently displaced from Golgi upon apoptotic stimuli (Fig. 4B, compare left and right panels). Induced apoptosis by staurosporine implies the cross-talk between two apoptotic pathways and is accelerated when caspase 3 is expressed in MCF-7 cells [25,26]. This mechanism is mediated by translocation of BAX to the mitochondria and the release of cytochrome c. However, DRO1 did not shuttle to mitochondria under these conditions but was instead retained

in the endoplasmic reticulum (Fig. 4B), resembling the collapsed Golgi and suggesting an endoplasmic reticulum stress-induced apoptosis. To further analyze the contribution of DRO1 repression in AIB1-mediated inhibition of apoptosis we silenced AIB1 alone or together with DRO1 and further treated the cells with staurosporine. As expected, silencing AIB1 resulted in increased apoptosis which was extensively impeded by silencing DRO1 as well (Fig. 4C). Although other pathways may also be relevant to explain AIB1-dependent inhibition of apoptosis (i.e. overexpression of IGF-I), our data reveals DRO1 pathway as a relevant target of AIB1 to prevent apoptosis and provide a novel mechanism to explain AIB1 as an oncogene.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2011.08.025.

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